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Highly effective gold nanoparticle-enhanced biosensor array on the wettability controlled substrate by wiping

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We demonstrate the use of a highly effective biosensor array to fulfill the requirements of high intensity, reduced nonspecific adsorption (NSA), and low sample usage. The mixed self-assembled monolayers (SAMs), consisting of methyl-terminated and methoxy-(polyethylene glycol (PEG))-terminated silanes, were newly applied as the background layer to reduce the background NSA via wettability control. The surface was modified by a plasma process with a pattern mask. Gold nanoparticles (AuNPs) were grafted within pattern-modified regions to increase intensity and were modified with protein G variants with cysteine residues to immobilize the antibody proteins directly. The target protein samples were selectively dewetted by the high throughput wiping process, while retaining semi-contact with the substrate. The data revealed that the background NSA was significantly reduced by 78% with selective dewetting compared to the standard method. Furthermore, the peak intensity was improved 5 times by applying AuNPs as compared to that of a planar surface, and the protein requirement was significantly reduced versus the standard process. © *2011 American Institute of Physics*. [doi:10.1063/1.3652860]

I. INTRODUCTION

Biosensors have received tremendous attention for use in a wide variety of applications, from fundamental cell/protein biology to industrial food processing. The use of microarray biosensors of biological molecules, such as antibodies, enzymes, and DNA, is a crucial technique for high throughput screening and fast analysis. Protein patterning requires the selective attachment of proteins at the desired region with concomitant prevention of nonspecific adsorption (NSA) at other regions. The self-assembled monolayer (SAM) is efficiently used as a biochip surface because SAM offers the desired surface wettability (hydrophobic/hydrophilic) as well as proper functional groups to immobilize proteins.[1–5]

Among the current methods of biological array patterning, such as micro-contact printing,[6] particle lithography,[7] and photolithography,[2,4] the soft lithographic method using an elastomeric stamp is one of the most versatile approaches and can satisfy the requirements of high throughput and low cost. However, this technique has major problems, such as stamp deformation and lateral diffusion of the solution.[6] To prevent NSA, protein-repellent surfaces, including SAMs with polyethylene glycol (PEG) or a hydroxyl (-OH) as a hydrophilic functional group, have been proposed for microarrays constructed on a Si or gold substrate.[3,5,8] There has been increased interest in PEG SAM for minimizing the interaction of proteins with the substrate and thereby suppressing biofouling due to the highly hydrated PEG chains.[9,10] In the specific binding region, a hydrophilic NH_2-terminated or COOH-terminated SAM has been widely used

with various cross-linking steps for covalent bonding with proteins on a planar substrate.[2,3,11] But the use of a planar substrate limits the number of biomolecules that can be attached on the specific region. A non-planar micro/nano-structure[12] or silica micro/nanoparticle[13] can be applied on the specific region to enhance the biomolecular-binding capacity and the detection sensitivity.

However, there are limitations in the application of previously developed compositions of specific and nonspecific layers for grafting proteins. Owing to the hydrophilic characteristics of both specific and nonspecific regions, a protein solution is generally immobilized by one of the following methods: The protein solution is dropped over the entire substrate,[5,8] the protein solution is dropped and covered with a coverslip,[14] or the substrate is dipped in protein solution during the protein reaction time.[4] With each of these methods, protein contact with the background layer is unavoidable, and background NSA occurs, even when PEG SAM is applied as the background layer.[15] Additionally, these processes are passive approaches to reduce NSA, and are inefficient in terms of sample usage. The ideal solution for preventing NSA would be a substrate that causes selective dewetting of protein solutions in specific regions; such a substrate would also provide the advantage of minimum sample requirement. Recently, due to their favorable optical, electronic, and chemical properties, gold nanoparticles (AuNPs) have been applied to enhance the binding of chemicals and the sensitivity of optical measurements. Through their affinity with functional groups, such as SH, CN, or NH_2, presented on the SAM surface, AuNPs are well suited to various biosensor applications.[4,16,17] Development of AuNPs-enhanced bio-applications has been primarily focused on

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optimizing chemical-binding affinity or the optical properties for measurement. However, in the development of biosensor array applications, not only chemical properties but also the physical morphology of specific and nonspecific regions must be simultaneously considered for enhancing sensitivity.

In this paper, we propose a new method for effective biosensor array fabrication with selectively grafted AuNPs on a wettability- and functional group-controlled substrate (Fig. 1). The SAM substrate was modified using oxygen plasma with a pattern mask for local conversion of the terminal groups. In the specific region, AuNPs, modified by protein G variants with cysteine residues, formed direct covalent bonds with an antibody, and the AuNPs-grafted non-planar surface enhanced the loading capacity and detection sensitivity. In the background region, the combination of methyl-terminated and methoxy-terminated PEG SAMs provided hydrophobicity to allow selective dewetting of the protein solution at specific versus nonspecific regions. A new semi-contact wiping process was used to apply the grafting protein sample, resulting in enhanced intensity and reduced sample usage.

II. MATERIALS AND METHODS

A. Materials

The CH$_3$-terminated octadecyltrichlorosilane [OTS; CH$_3$(CH$_2$)$_{17}$SiCl$_3$] and OCH$_3$-terminated 2-[methoxy(polyethyleneoxy)propyl]trichlorosilane [PEG; CH$_3$O(C$_2$H$_4$O)$_{6-9}$(CH$_2$)$_3$SiCl$_3$] SAMs were from Gelest. The NH$_2$-terminated 3-aminopropyltrimethoxysilane [APTMS; H$_2$N(CH$_2$)$_3$Si(OCH$_3$)$_3$] for adsorbing the AuNPs was from Aldrich. The Protein G variant with cysteine residues (cys-protein G) was engineered by MiCoBioMed in Korea Research Institute of Bioscience and Biotechnology. Protein G for immobilizing the antibody, glutaraldehyde (GA), the cross-linker between the NH$_2$ functional group and the protein G, and Tween 20, a non-ionic detergent, were from Aldrich. All chemicals were used without additional purification.

B. Plasma-induced array pattern modification

Figure 1(b) shows the pattern modification procedure. A glass slide (25 × 75 mm) was prepared by immersing it in piranha solution (1:3 H$_2$O$_2$:H$_2$SO$_4$) at 80 °C for 30 min and rinsed in de-ionized (DI) water and dried in a stream of nitrogen gas (N$_2$). The substrate was immersed in anhydrous toluene containing 2 mM OTS and PEG SAMs for 30 min and sonicated for 10 min in toluene and washed with acetone, methanol, and DI water. Mixing molar ratios of OTS/PEG of 3:1, 1:1, and 1:3 were fabricated using the same process.

For the SAM patterning, the treated glass substrate was loaded in the plasma chamber (plasma finish, V15-G) with an invar pattern mask for local conversion of the terminal group.[1] Given that the ionized atoms to modify the SAM layer and the radicals for surface activation in the plasma state have no directivity, the gap between the substrate and the pattern mask should be minimized to avoid pattern broadening. Consequently, an invar mask and a neodymium magnet were used to generate the magnetic force, and a glycerol layer was coated between the mask and the glass substrate to reduce the gap. The oxygen plasma was exposed at 500 W power for 90 s to modify the surface. The local conversion of the terminal functional group to the hydroxyl radicals occurs for cross-linking with organosilane SAMs. The used hard mask is not deformed, and thus the pattern mask is reusable. In addition, this allows the fabrication of many SAM templates at the same time in a plasma chamber, thereby satisfying the high throughput and low cost criteria.

The selectively modified sample was immersed into 1% APTMS solution for 4 h. As a result, the locally modified OTS regions could be grafted with NH$_2$-terminal groups of APTMS while the unmodified OTS regions remain with the original surface characteristics.

C. Adsorption of AuNPs

The Au colloid was prepared by adding 60 mg of HAuCl$_4$ to 125 mL of de-ionized (DI) water, which was then
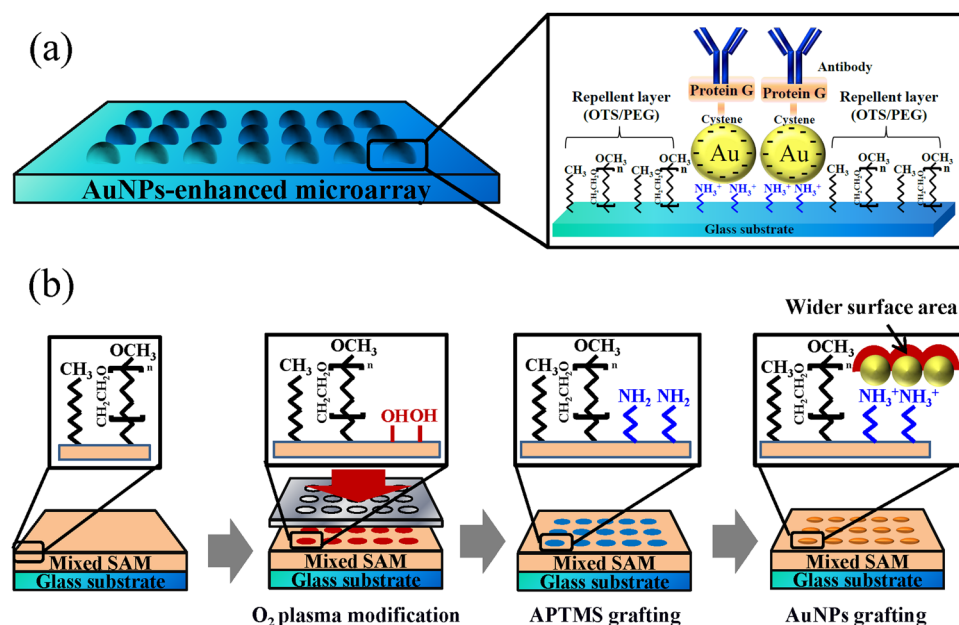

FIG. 1. (Color online) (a) Concept of proposed biosensor array chip. (b) Chip fabrication procedure.

boiled at 250 °C. Then, sodium citrate solution (25 mL containing 250 mg) was added and boiled for 5 min.[18] The average diameter of the particles produced was 13 ± 2 nm, as determined by transmission electron microscopy. The Au colloid solution was dispensed on the plasma pattern modified substrate and washed with DI water 10 times. When the Au colloid solution wetted the APTMS layer, the APTMS terminal functional group was protonated ($-NH_2 \rightarrow -NH_3^+$) in the suspension. Thus, negatively charged Au NPs were assembled onto the positively charged amino groups by electrostatic interactions.

D. Protein grafting by wiping process

The wiping module was fabricated to control the gap between the substrate and wiping glass using a micrometer. The wiping speed was set at 2 mm/s, and the gap between the substrate and wiping glass was fixed at 100 μm as shown in Fig. 2(a).

The antibody array was fabricated on the selectively modified substrate, and whole proteins including AuNPs were grafted by wiping process as shown in Fig. 2(b). Cys-protein G was assembled on the Au NP layer to immobilize the antibody directly. Cys-protein G has superior antibody binding ability and forms a properly oriented protein layer
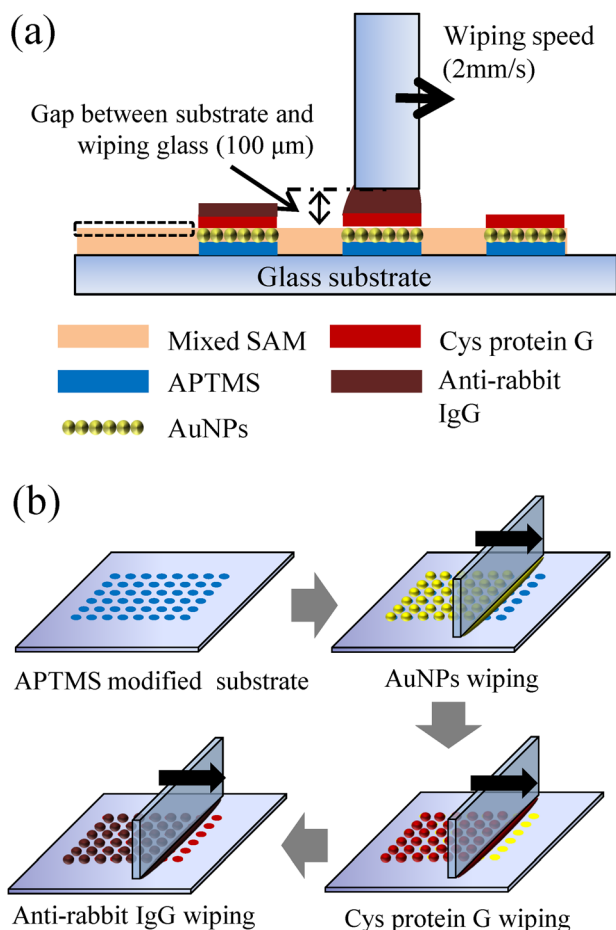

FIG. 2. (Color online) (a) Wiping procedure on a patterned substrate (mixed OTS/PEG SAM: background layer; AuNPs-grafted layer: specific region). (b) Selective dewetting of protein solution sequentially on patterned chip by wiping process.

through thiol group adsorption on the gold surface.[19] To add the protein layer, cys-protein G (1 mg/mL) in phosphate-buffered saline (PBS) was dispensed on the treated substrate for 1 h at 25 °C, and the modified substrate was washed with the non-ionic detergent Tween 20 (0.05%, v/v, in PBS). Cyanine 3 (Cy3)-labeled anti-rabbit immunoglobulin G (IgG, 10 μg/mL in PBS) was used as a target protein. The Cy3-labeled antibody was incubated at 4 °C for 1 h in a moisture chamber, and the array was washed with PBS and DI water to remove the physically absorbed antibodies. The experiment was performed with an uncharged SAM with the pH of the buffer solution fixed at 7.4 to avoid other effects of protein adsorption, such as charge-charge interactions.[20]

E. Wettability and intensity measurement

The contact angles were measured using a DSA 100 device (Krüss). The static contact angle was measured by a 5 -μL droplet of DI water. Protein adsorption on the glass substrate was analyzed with a GenePix Pro fluorescence scanner with a fixed photomultiplier tube (PMT; gain, 350; power, 80%).

III. RESULTS AND DISCUSSIONS

A. Background wettability and protein adsorption

The contact angles of DI water on the mixed OTS/PEG SAMs layer were measured to analyze protein adsorption according to the wettability and to determine the contact angle differences between the specific and background regions for the selective dewetting condition (Fig. 3). The contact angle of DI water on the mixed OTS/PEG layer varied with the mixing ratio, and decreased linearly, from 105° to 37°, with the mole fraction of PEG. This decrease was expected because OTS, which has a hydrophobic methyl functional group, was replaced by PEG, which has a hydrophilic methoxy end group. For SAM with 0% and 100% PEG SAM contents, the contact angle deviation was small and indicated that almost completely homogeneous hydrophobic and hydrophilic surfaces were obtained. Although the contact angle deviations of heterogeneous SAMs (25%, 50%, and 75%) were larger than those of homogeneous SAM, owing to the random ordering of mixed SAMs,[21] the
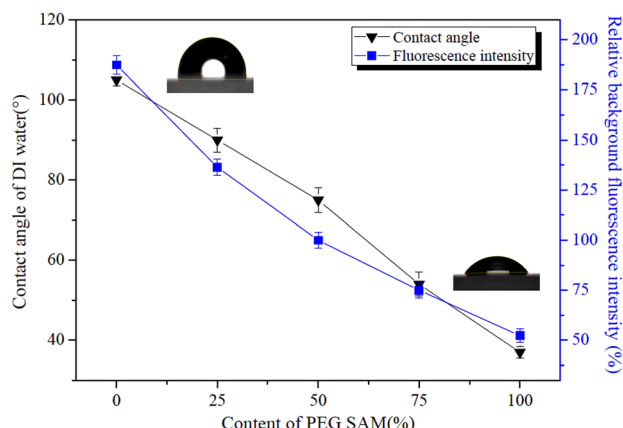

FIG. 3. (Color online) Protein adsorption according to the substrate wettability (mixing ratios of OTS/PEG SAMs).
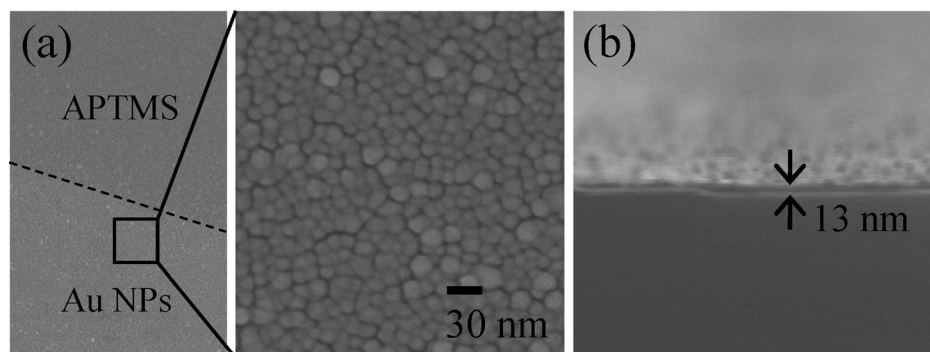
FIG. 4. SEM images of assembled AuNPs: (a) Surface topology of the AuNP layer and the unmodified layer at the pattern boundary. (b) Cross-sectional SEM image of the AuNPs layer.

deviation was slight (approximately $\pm 3°$). Therefore, the mixed SAM layer was successfully prepared on the glass substrate. This means that wettability can be controlled by mixing ratios of hydrophobic/hydrophilic SAMs. This layer can be applied to various protein immobilizations by wiping.

Cy-3-labeled anti-rabbit IgG adsorption was examined according to the OTS/PEG SAM mixing ratios. All experiments were performed under the same conditions using the standard method for assessing the relationship between wettability and protein adsorption. Fluorescence intensity was compared with the same PMT (gain, 350). Background intensity data were measured by the line profile along with the pattern. Intensity levels decreased linearly with an increasing mole fraction of PEG SAM. The protein adsorption was affected by wettability and functional group.[22,23] Because of the hydrophobic interaction between the protein and the substrate,[24,25] the protein adhered more to the hydrophobic surface than to the hydrophilic surface in many cases. Two different kinds of functional group-terminated SAM, which have opposite protein adsorption and wettability characteristics, were mixed. Results indicated that protein adsorption was linearly related to the mole fraction of mixed SAM (wettability).

B. AuNPs-enhanced array chip

Using the plasma-inducted modification method, the array pattern was successfully fabricated on the mixed SAM glass substrate and AuNPs were grafted on the pattern region, as shown in Fig. 4(a). The AuNPs could be grafted using 2 methods: They could be immersed into the Au colloid solution or selectively dewetted by the wiping process. To avoid the undesired adsorption as well as to reduce the usage of colloidal solution, the AuNPs were grafted by the latter method.

DI water on the APTMS layer had a hydrophilic contact angle of $43° \pm 2°$. For the selective dewetting, the wettability of the background layer was controlled by changing the OTS/PEG ratio. The contact angles of DI water on the mixed SAM substrate were measured as mentioned above. From a 50% content of PEG SAM (mol%), the AuNP colloid solution could be selectively dewetted on the specific region owing to the contact angle differences and the high surface tension (71.97 mN/m) of the AuNP colloid solution. The wiping procedure was done in a high humidity chamber to suppress solution evaporation. The AuNPs were wiped

several times to ensure close packing of AuNPs, avoid non-grafted regions, and minimize nonspecific binding on the glass, as shown in Fig. 4(a). To investigate the depth of grafted AuNPs, the cross-sectional image of AuNP-grafted region was measured with SEM, as shown in Fig. 4(b). It was determined that the grafted AuNPs formed monolayers.

C. Wettability of the specific region and selective dewetting condition

The selective dewetting condition was determined by the surface tension of the solution and the surface energy of the substrate.[26] PBS buffer was used as a protein solvent. The surface tension of PBS buffer is 71.4 mN/m at room temperature,[27] which is almost the same as that of DI water (71.97 mN/m) at room temperature.

The cys-protein G (1 mg/mL) and Cy3-labeled anti-rabbit IgG (10 μg/mL) were sequentially dispensed via the wiping process. Because the protein played the role of the surfactant, if the concentration of the protein solution was increased, the surface tension of the solution was reduced. To overcome this phenomenon and to sustain the high surface tension of the solution, cys-protein G and Cy3-labeled anti-rabbit IgG stocks were stored at 2 °C and a low temperature was maintained as they were applied by the wiping process; surface tension generally increases with a decrease in temperature owing to increased attraction between molecules.[28] To verify the temperature effect according to the concentration of proteins, the contact angles of DI water, PBS buffer, cys-protein G (1 mg/mL), and Cy3-labeled anti-rabbit IgG (10 μg/mL) were measured on the 1:1 OTS/PEG SAM substrate. The contact angle change in the low-temperature (2 °C) protein solution on the substrate was slight, within $5° \pm 2°$, compared with the contact angle of PBS buffer at 25 °C. Thus, the protein solution could be selectively wetted on the hydrophilic region because of the high polarity of the solution.

Figure 5 shows the contact angles of the specific and nonspecific regions. The contact angle of DI water on the AuNPs-grafted layer (specific region) was fixed as $55° \pm 2°$, that of the cys-protein G immobilized layer was $45 \pm 2°$, and that of the nonspecific region was controlled. In the case of 75% PEG SAM content, the contact angles of the specific and nonspecific regions were almost identical on the AuNPs-grafted layer. From a 50% content of PEG SAM (mol%), selective dewetting of protein solution was achieved without protein solution residues (smearing defects outside the
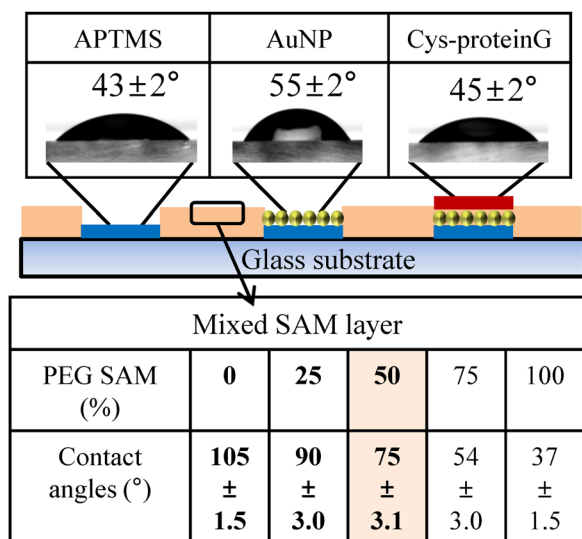
FIG. 5. (Color online) Contact angles of DI water on the specific region and non-specific region.

pattern) due to the difference in the contact angle[29] between the specific region and the background region.

D. Effects of selective dewetting on mixed SAM substrate

Protein adsorption was affected by surface wettability and the functional group of the substrate. Additionally, protein adsorption is increased by increasing the concentration of protein solution and reaction time with the substrate.[30] The effect of the selective dewetting method is minimization of the contact (reaction time) of the protein solution with the background layer.

The protein (Cy-3-labeled anti-rabbit IgG, 10 μg/mL) adsorption of the background layer was measured and compared between the selective dewetting method by wiping and the standard method with a coverslip, according to the mixing ratios of OTS/PEG SAM as shown in Fig. 6. The intensity profiles obtained with both the wiping and standard methods decreased with an increasing mole fraction of PEG
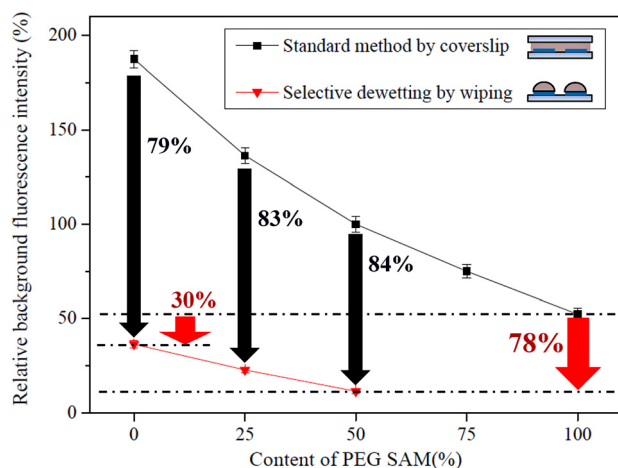

FIG. 6. (Color online) Background NSA comparison according to the OTS/PEG mixing ratios: Standard method and selective dewetting method.

SAM. Although PEG SAM is a well-known biofouling layer, adsorption in the OTS layer was reduced by 30% by use of the wiping method compared with the PEG layer, indicating the effectiveness of the selective dewetting method. Under the condition of 50% content PEG SAM (mol %), the background adsorption was reduced by 78% by use of the wiping method compared with a PEG SAM layer by the standard method. It is expected that in the standard method, the protein was in contact with the background region during the full reaction time (30 min). However, by applying the selective dewetting method, the protein adsorption on the background occurred only during the wiping process (a few seconds). The wiping speed was 2 mm/s, and the linewidth of the wetted area during the wiping process was about 1 mm (the size of the wiping glass side); thus, the contact time between the protein solution and the background layer can be estimated as less than 1 s. As a result, the background NSA was significantly reduced by applying the selective dewetting method.

E. Effects of non-planar specific region obtained by applying AuNPs

The surface area can be increased by applying a non-planar substrate rather than a planar substrate. Therefore, high molecular density arrays could be fabricated by applying AuNPs. The amount of increase in the surface area was calculated by considering that each closely packed particle occupies a hexagonal area on the surface. The non-planar surface area created by closely packed particles is approximately 1.813 times higher than a planar surface,[31] regardless of the size of the particles.

To experimentally verify the roughness of a specific surface, the surface topology was measured by atomic force microscopy, and various roughness parameters, such as the mean roughness (R_a), root mean square roughness (R_{rms}), and 10-point mean roughness (R_z), were compared between a non-planar surface and the planar layer, as shown in Fig. 7(a). Figure 7(b) shows the line profile comparison between the AuNPs and APTMS surfaces. The APTMS surface had the lowest values for various roughness parameters, while the AuNP-grafted surface exhibited substantially higher roughness, indicating that a non-planar surface with a high roughness factor can be obtained by applying nanoparticles.

To compare the protein adsorption according to surface topology, the planar substrate was fabricated by applying protein G on the glass. On the APTMS region, glutaraldehyde (GA) activation for cross-linking was performed in a 10% solution of GA in PBS solution at 25 °C for 1 h to reduce the bridging and maximize the yield. Protein G (1 mg/mL) in PBS buffer was dispensed on the treated samples for 1 h at 25 °C and was washed with PBS-T. Cy3-labeled anti-rabbit IgG (10 μg/mL) was dispensed in the same manner as in the AuNPs-enhanced chip experiment.

The contact angle of DI water on the GA-coated layer was fixed as 62° ± 2° and that of the protein G grafted layer was 55° ± 2°. Therefore, from a 50% content of PEG SAM (mol%), selective dewetting of streptavidin solution was achieved equal to AuNPs-enhanced chip fabrication.
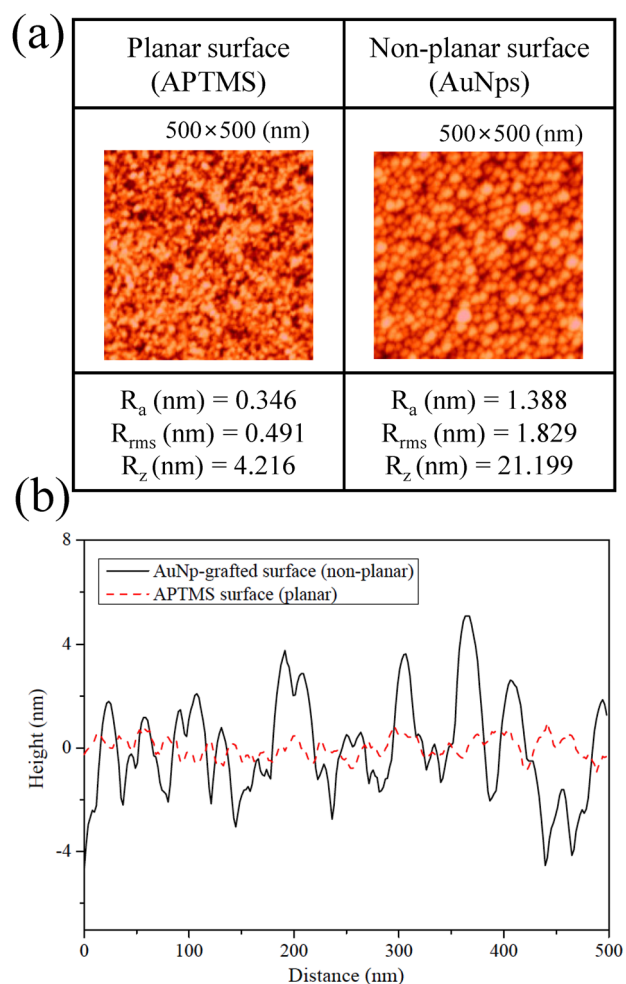
FIG. 7. (Color online) (a) Surface topology of planar and non-planar substrate, and surface roughness. (b) Line profile comparison between AuNPs surface and planar surface.

A. Planar surface by standard method

B. Planar surface by wiping method

C. Non-planar surface by standard method

D. Non-planar surface by wiping method

FIG. 8. (Color online) Average peak intensity of specific region comparison according to substrate topology and protein grafting method.

Figure 8 shows the average peak intensities. In total, peak intensities from 4 different methods were compared: (A) standard method using a coverslip on a planar substrate chip with a PEG SAM background, (B) selective dewetting on a planar substrate chip with a 50% content PEG SAM background, (C) standard method using a coverslip on an AuNPs-enhanced chip with a PEG SAM background, and (D) selective dewetting on an AuNPs-enhanced chip with a 50% content PEG SAM background. The effects of selective dewetting were observed by comparing A with B and C with D. The peak intensity obtained by the selectively dewetting method was increased by 20% compared to that of the standard method. It is assumed that this is due to the difference in the droplet volume of the protein solution on the specific region. In the standard method, the area between the coverslip and substrate was filled with the protein solution. However, in the selective dewetting method, the protein solution was maintained by droplet formation on the specific region owing to the differences in wettability between the specific and nonspecific regions. Thus, the possibility of reaction activity was increased owing to the total increase in the amount of mole.

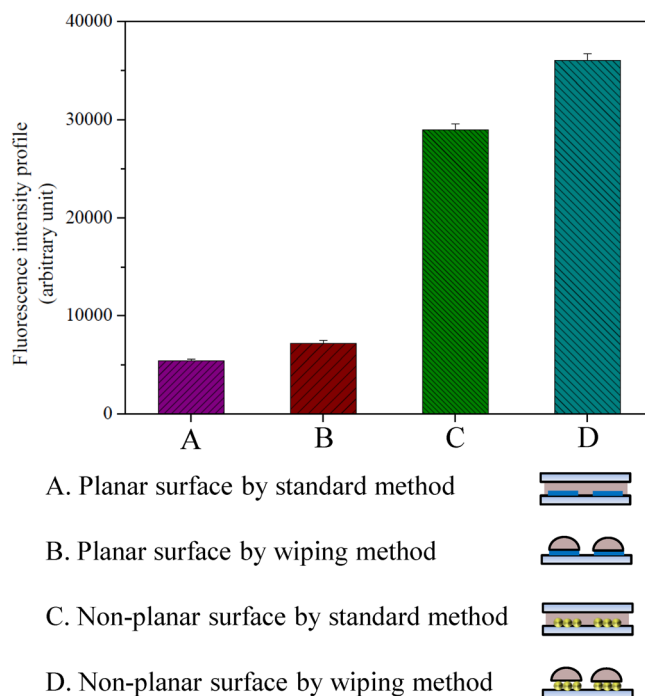The change in surface topology also led to a change in peak intensity; the peak intensity on that nanoparticle-modified surface was increased by about 5 times compared to that on the planar surface. This experimental increase was much higher than the theoretical increase of the surface area. The reason could be that, when performing surface modification on nanoparticles in solution, the chemical reaction efficiency of the binding proteins is higher than that conducted on the planar substrate.[32] As the dimensions of the antibody are in the nanometer range, a surface with nanotopological features similar to or smaller than the size of the protein may be sensed by the protein and affect its behavior and binding affinity.[33]

F. Reduction of protein usage by selective dewetting process

The protein usage was also reduced by application of the selective dewetting method by wiping. Well-known standard methods for protein grafting include dipping, dropping, and the process of covering the sample with a coverslip. The dipping and dropping methods require large amounts of protein solution to cover the entire substrate; thus, the coverslip method is preferred to reduce protein usage. The protein solution is dropped onto the substrate and covered with a coverslip for the reaction to occur over the entire substrate. This method requires a solution of approximately 3–5 μL for each coverslip (general size = 18×18 mm). A fixed volume of protein was required per covered area in the standard method.

However, by applying the wiping method, the entire microscope glass slide (25×75 mm) could be selectively coated with the same (3–5 μL) amount of protein and the solution remained after the wiping process was complete. On a 100 μm spot array, if the droplet formed is hypothesized to be a hemisphere, the droplet volume of a 100-μm diameter spot was 261.79 pL. Therefore, with 3 μL protein solution, approximately 11 449 array pattern could be fabricated. This
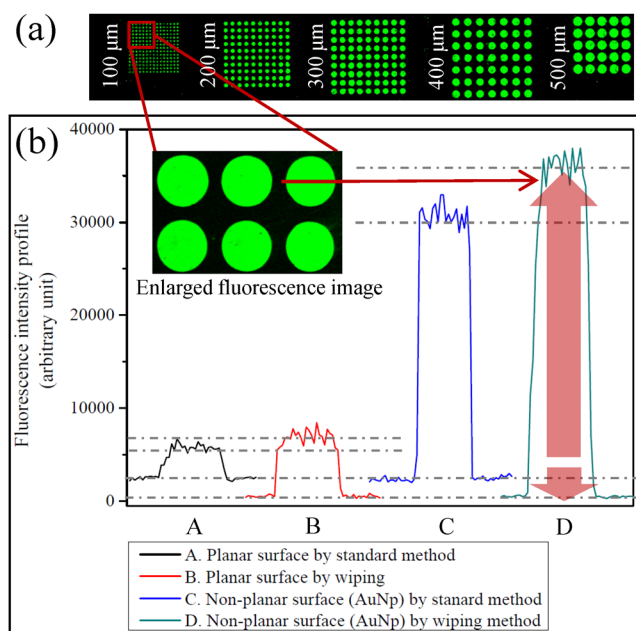
FIG. 9. (Color online) (a) Fluorescence labeled antibody array from $100\,\mu m$ to $500\,\mu m$ diameter on the AuNPs-grafted substrate by wiping process. (b) Fluorescence intensity profile comparison according to substrate topology and protein grafting method.

means that by using the wiping process, the droplet volume can be controlled to cover a given number and size of arrays without wasting protein solution. Thus, by reducing NSA, the wiping process can be applied on a large substrate with a small amount of protein.

G. Intensity profiles of array chips

Array pattern chips were fabricated using the proposed wiping method on an AuNPs-enhanced chip. Uniform Cy3-fluorescence spots with diameters of 100 to $500\,\mu m$ were successfully fabricated on the whole substrate of glass slide $(25 \times 75\,mm)$ with the wiping process as shown in Fig. 9(a). The Cy3 dyes emitted green fluorescence at $570\,nm$ when excited at $550\,nm$. To verify the edge shape of spot, the $100\,\mu m$ magnified image was measured by confocal microscope, and the clean spot edge was achieved as shown in Fig. 9(b). The line profile and pattern were measured to investigate the peak and ground levels. As mentioned above, the peak and ground intensity profiles were analyzed for 4 types of arrays. The AuNPs-grafted array chip showed the highest peak intensity and lowest ground intensity. The increased surface area of this chip will subsequently increase the density of biomolecules that can be immobilized and the sensitivity for detection. Furthermore, the minimal contact of proteins with the background by the wiping method could effectively reduce the NSA and enhance the detection sensitivity. Finally, the high intensity and reduced NSA array chip can be fabricated using the selective dewetting method on the wettability-controlled substrate by grafting AuNPs on the specific region.

IV. CONCLUSIONS

In this study, we proposed and tested a high-intensity and low-noise protein array sensor that was prepared by

selectively grafting AuNPs on a pattern-modified substrate. The background layer was improved by using a mixed SAM to control selective wettability and to reduce nonspecific binding by 78% without the need for any additional blocking process. In the specific region, the non-planar pattern was applied using cysteine-tagged protein G-modified AuNPs that enlarged surface area and directly immobilized the antibody with proper orientation. By this method, the efficiency of full-coverage adsorption was increased by more than 5 times. Additionally, the proposed semi-contact wiping process was a fast and simultaneous process that efficiently reduced NSA and sample usage. We believe that this method will contribute to advances in low-cost, high-sensitivity, and high-throughput biosensor array applications.

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